METHOD FOR DETERMINING PLASMINOGEN ACTIVATOR INHIBITOR

Field of the Invention

This invention relates to the determination of the level of active plasminogen activator inhibitor Type 1 in samples such as biological fluids.

Background of the Invention

In order to ensure an adequate blood supply to various organs, the mammalian body is equipped with two systems, a coagulation system and a fibrinolytic system. The coagulation system functions to stop bleeding and protect the mammal from blood loss. The fibrinolytic system functions primarily to dissolve blood clots. The two systems are normally in equilibrium and the enzymes involved in both systems are under control at multiple levels.

The key enzyme of the fibrinolytic system is plasmin, which digests the fibrin threads of a fibrin blood clot. Plasmin is formed when its precursor protein, plasminogen, is activated by a plasminogen activator. Plasminogen activators are typical serine proteases and four different plasminogen activator (PA) systems are recognized; (a) factor XII-dependent system, (b) streptokinase (isolated from Streptococci), (c) tissue plasminogen activator (tPA) and (d) urinary plasminogen activator (urokinase or uPA). In humans, only tPA and uPA have physiological importance, tPA being the main fibrinolytic enzyme in the circulation.

The plasminogen activating activity of tPA and uPA is inhibited by several plasminogen activator inhibitors (PAI). Four types of PAI have been described: (a) endothelial-type inhibitor (called Plasminogen Activator Inhibitor Type 1 or PAI-1); (b) placental inhibitor (called Plasminogen Activator Inhibitor Type 2 or PAI-2); (c) heparin-dependent inhibitor (Plasminogen Activator Inhibitor Type 3); and (d) the protease nexin (Plasminogen Activator Inhibitor Type 4) (Urden et al., (1987), Thromb. Haemost., v. 57, pp. 29-34; Francis et al., (1988), Am. Heart J., v. 115, pp. 776-780; and Kurnik, (1995), Circulation, v. 91, pp. 1341-1346).

the nature of the interaction of PAI-1 and vitronectin remains the subject of considerable debate.

Numerous clinical reports have documented that failure of the endogenous fibrinolytic capacity is attributable to an increase in serum PAI-1 activity. Stringer et al., (1994), Arterioscler. Thromb., v. 14, pp. 1452-1458, reported that PAI-1 is released at high concentration from activated platelets and is retained within the thrombus by binding to fibrin, resulting in inhibition of local tPA-mediated clot-lysis. Furthermore, the administration of monoclonal antibodies that block the inhibitory activity of PAI-1 reduced clot lysis resistance. In patients with coronary artery disease (CAD), Hamsten et al., (1985), N. Eng. J. Med., v. 313, pp. 1557-1563, have documented that in young survivors of acute myocardial infarction (AMI), an elevated plasma level of PAI-1 up to 3 years after the event was correlated to a higher rate of reinfarction. Since this initial report, several other investigators have confirmed these observations.

The plasma active PAI-1 level was also investigated, and reported elevated, during the acute coronary thrombotic events. Furthermore, in patients with AMI, the plasma level of PAI-1 was correlated with the capacity to lyse a coronary thrombus. In patients who fail to have restored coronary blood flow, as evident by coronary angiography (determined by angiography 24 hr-1 week after AMI) or by the development of a Q-wave on the ECG, a high plasma level of PAI-1 was documented (Sakamoto et al., (1992), Am. J. Cardiol., v. 70, pp. 271-276 and Ogava, (1993), Cardiol., v. 41, pp. 201-208). From the several studies reported, it can be concluded that in patients with CAD, a high plasma level of PAI-1 is associated with a high risk for developing acute coronary ischemia and that in those who develop an acute event, a high plasma active PAI-1 level is associated with an ominous outcome.

To further establish the role of a balanced equilibrium state between tPA and PAI-1 activities in native fibrinolysis, several clinical trials have investigated patient outcome in artificially induced endothelial dysfunction. In patients who were subjected to Percutaneous Transluminal Coronary

Several direct and indirect functional methods to quantify the fibrinolytic inhibition capacity of biological samples have been described. (Verheijen et al., U.S. Patent No. 4,563,420; Pussard et al., U.S. Patent No. 5,472,851; Sasamata et al., U.S. Patent No. 5,102,787). The most commonly used method, Verheijen et al., (1985), Thromb. Res., v. 39, pp. 281-8, measures inhibition of tPA activity, which is primarily due to PAI-1 activity, through the hydrolysis of either a tPA-specific substrate or a plasmin-specific substrate, plasmin having been produced by the action of tPA upon plasminogen. This hydrolysis results in either a measurable chromogenic change or in the breakdown of a fibrin film resulting in measurable clot lysis.

The European Committee of Fibrinolysis evaluated the various functional methods available for measuring tPA inhibition in a multicentre study and concluded that they have limited accuracy, Gram et al., (1993), Thrombosis and Haemostasis, v. 70, pp. 852-857. The main drawbacks of these methods are the presence of a partitioning step of the plasma euglobins, the non-standardization of the incubation conditions, and of the form and amount of tPA to be utilized and the indirectness of measurements. Also, some of these methods discount the role of plasmin inhibitor activities in the test samples. Another problem encountered in methods of measuring inhibition of tPA functionally is the fact that the activities of both tPA and PAI-1 are unstable and decrease gradually after sample collection. In blood with high PAI-1 levels, the tPA activity can decrease by 50% in about one minute.

In order to avoid the problems encountered with functional assay methods for measuring active PAI-1, several immunoassay methods have been developed. The simplest assays employ an antibody to PAI-1 in a conventional immunoassay (for example, U.S. Patents Nos. 5,422,245 and 5,629,160). Methods have also been described for measuring active PAI-1 by a two-step procedure: the sample under investigation is divided into two portions and a saturating amount of tPA is added to one portion. The level of PAI-1/tPA complex is then measured in both portions. The difference in the measured amount of the PAI-1/tPA complex between the two portions represents the amount of free or active PAI-1.

The method of the invention determines the level of active PAI-1 in circulation by determining the amount of PAI-1 complexed to multimeric vitronectin.

The present invention provides an improved method for determining active PAI-1. The method is much less cumbersome than methods involving comparison of PAI-1/tPA complex levels with and without addition of exogenous tPA. The present method, which measures active PAI-1 directly, as the stable PAI-1/multimeric vitronectin complex, is also less subject to interference from uncontrolled factors such as inconsistencies and artifacts of tPA binding than previously described methods for determining plasma active PAI-1.

In accordance with one embodiment of the invention, a method for determining active plasminogen activator inhibitor-Type I (PAI-1) in a biological fluid comprises the steps:

(i) providing a sample of a biological fluid; and

(ii) measuring the amount of PAI-1/multimeric vitronectin complex in the sample to determine active PAI-1 in the sample.

The biological fluid to be assayed may be selected from the group consisting of whole blood, plasma, serum, saliva, amniotic fluid, cerebrospinal fluid, tissue extract or urine.

In accordance with a further embodiment, a kit for determining active PAI-1 in a biological fluid comprises:

(a) a first antibody which binds selectively to PAI-1; and

(b) a labelled second antibody which binds selectively to multimeric vitronectin.

Detailed Description of the Invention

The present invention provides a method for determining active PAI-1 in a biological fluid by determining the amount of PAI-1/multimeric vitronectin complex present in the fluid.

of the amount of PAI-1/multimeric vitronectin complex present in the sample and hence determination of active PAI-1 in the sample.

In accordance with a further embodiment, the sample is contacted with a first antibody which binds selectively to multimeric vitronectin and does not bind substantially to monomeric vitronectin. The first antibody carries a detectable label or a component of a signal-generating system. The sample is then contacted with a second antibody which binds selectively to PAI-1. Determination of the PAI-1/multimeric vitronectin complex, and of active PAI-1, is as described above.

The first and second antibodies may be added separately in a two-step procedure or may be added simultaneously.

Active PAI-1 may be determined as PAI-1/multimeric vitronectin complex by the method of the invention in a biological fluid such as whole blood, plasma, serum, urine, saliva, cerebrospinal fluid, amniotic fluid or a tissue extract.

The biological fluid is preferably whole blood, plasma or serum. When blood is collected for assay of active PAI-1 in whole blood, serum or plasma, care must be taken to avoid platelet activation, for example by using citrate as anticoagulant or by employing special blood collection tubes which promote platelet stabilisation and avoid platelet activation during blood collection; examples of suitable commercially available tubes are Stabilyte™ Blood Collection tubes, available from American Diagnostica Inc., and Becton Dickinson tubes, Catalog No. 6457.

The anti-PAI-1 antibodies used in the methods of the invention should be able to recognise PAI-1 when it is bound to multimeric vitronectin. They should therefore be directed against PAI-1 epitopes which remain exposed in the active PAI-1/vitronectin complex.

The anti-multimeric vitronectin antibodies used should recognise multimeric but not monomeric vitronectin. They should therefore be directed against epitopes exposed in multimeric vitronectin but not accessible in monomeric vitronectin. It is believed that the unique epitopes exposed in denatured vitronectin will also be present in the multimeric vitronectin of the

adjuvant (complete and incomplete), aluminum hydroxide, surface-active substances such as lysolecithin, polyanions, emulsions of oil and keyhole limpet hemocyanins.

Monoclonal anti-PAI-1 or anti-multimeric vitronectin antibodies may also be produced by methods well known in the art. Briefly, the purified protein is injected in Freund's adjuvant into mice over a suitable period of time, spleen cells are harvested and these are fused with a permanently growing myeloma partner and the resultant hybridomas are screened to identify cells producing the desired antibody with the required binding selectivity. Suitable methods for antibody preparation may be found in standard texts such as Antibody Engineering, 2d. edition, Barreback, Ed., Oxford University Press (1995).

Monoclonal antibodies produced by a selected hybridoma clone may be purified by known techniques such as ammonium sulfate fractionation, DEAE cellulose chromatography or affinity chromatography utilizing protein G or A- Sepharose column chromatography, cellulose membranes and agarose and synthetic materials such as cross-linked polysaccharides, polyvinylchloride, polypropylene, polystyrene and the like or their combinations.

Anti-PAI-1 antibodies displaying the desired binding specificity, as described above, may be obtained using screening methods similar to those described by Declerck et al., (1988), Blood, v. 71, p. 220, and anti-multimeric vitronectin antibodies may be screened for desired binding specificity as described by Sockman et al., (1993), v. 268, p. 22874 or Seiffert et al., (1994), J. Biol. Chem., v. 269, p. 2659.

The second antibody carries a label which may be any suitable directly detectable label or a component of any suitable signal-generating system. Many examples of these are well known from the field of immunoassay.

Labelling of the second antibody with a detectable label or a component of a signal-generating system may be carried out by techniques well known in the art. Examples of labels that can be utilized to render an antibody detectable include radioisotopes, enzymes, fluorescent and

size, polystyrene beads, polyacrylamide matrices, paramagnetic particles, latex particles or gelatin particles.

Antibodies may be immobilised on a solid support by conventional methods which are well known in the art, for example as described in U.S. Patent No. 5,352,583.

In accordance with a preferred embodiment of the invention, a sample of a biological fluid is contacted with a first antibody which binds selectively to PAI-1 to form a complex, the first antibody being immobilised on a solid support. Sufficient time is allowed to permit binding of the PAI-1 of the sample to the immobilised antibody. The solid support is then washed and contacted with a second antibody which binds selectively to multimeric vitronectin and is labelled with a detectable label or has attached to it a signal-generating system. The label or generated signal bound to the solid support is determined, providing a measure of the PAI-1/multimeric vitronectin complex present in the sample, and hence determining the level of active PAI-1.

In accordance with a more preferred embodiment, the sample is contacted simultaneously with the immobilised first antibody on the solid support and the labelled second antibody.

In a further embodiment, the second antibody may lack a label or signal-generating system component and the solid support-bound second antibody is determined by means of a third antibody bearing a detectable label or signal-generating system component, the third antibody binding selectively to the bound second antibody.

In accordance with a further embodiment, the sample is contacted, either simultaneously or stepwise, with a first antibody which binds selectively to PAI-1 and to which is attached one member of a capture pair and with a labelled second antibody which binds selectively to multimeric vitronectin. The resulting mixture is then contacted with a solid support on which is immobilised the other member of the capture pair. After allowing sufficient time for the labelled PAI-1/multimeric vitronectin complex to bind to the solid support by interaction of the members of the capture pair, the solid support is

In accordance with a further embodiment, the kit comprises (a) a first antibody which binds selectively to multimeric vitronectin and (b) a labelled second antibody which binds selectively to PAI-1 or a second antibody which binds selectively to PAI-1 and a labelled third antibody which binds selectively to the second antibody.

The anti-PAI-1 or anti-multimeric vitronectin first antibody may be immobilised on a solid support.

The kit may also contain a set of calibration standards. The kit may also optionally contain additional reagents such as diluents or buffers which are employed in the methods of the invention and calibration standards.

Examples

The examples are described for the purposes of illustration and are not intended to limit the scope of the invention.

Example 1

Reagents:

Coating buffer (CB):	40 mM K/phosphate buffer, pH 7.4 100 mM NaCl
Blocking buffer (BB):	40 mM K/phosphate buffer, pH 7.4 100 mM NaCl 1% hydrolysed casein
Incubation buffer (IB):	40 mM K/phosphate buffer, pH 7.4 100 mM NaCl 5 mM EDTA 1% hydrolyzed casein 0.025% Tween-20
Washing buffer (WB):	40 mM K/phosphate buffer, pH 7.4 100 mM NaCl 0.025% Tween-20

ELISA plates (Immulon II, Dynax)
First antibody: monoclonal anti-PAI-1 antibody
Second antibody: HRP-labelled anti-multimeric vitronectin antibody

microtitre plate are coated with 100 μL/well of CB containing anti-PAI-1 monoclonal antibody (5-15μg/ml). The plates are incubated at 4°C for 16-18 hours, the wells are washed three times with WB, blocked with 200 μL/well of BB for 1 hour and then washed three times with WB.

50 μL portions of the plasma samples under testing or of the various concentrations of the PAI-1-mVn complex standards (final concentration of PAI-1 in the PAI-1/mVn complex range from 0 to 100 ng/ml) are added to each well followed by 50 μL well of IB. The plates are then incubate at room temperature with shaking for 60 min. and, washed three times with WB.

100μL of HRP-labelled anti-mVn monoclonal antibody (2-5μg/ml) in IB is added to each well, the plates are then incubated at room temperature with shaking for 60 min, washed three times with WB and developed with the HRP substrate for 15 minutes according to the manufacturer's instructions.

The enzyme reaction is terminated by addition of 100 μL/well of concentrated sulfuric acid. The intensity of the resulting colour is determined by reading adsorbancy at 492 nm in the microtitre plate reader. The concentration of active PAI-1 in a sample is determined by comparison with the calibration curve.

Example 3

Reagents are as described in Example 1 except for the second antibody which is biotinylated and an HRP-conjugated Streptavidin detection system is utilized, to measure bound second antibody.

The wells of an ELISA microtitre plate are coated with 100 μL/well of CB containing anti-PAI-1 monoclonal antibody (5-15μg/ml). The plates are incubated at 4°C for 16-18 hours, the wells are washed three times with WB, blocked with 200 μL/well of BB for 1 hour and then washed three times with WB.

50 μL portions of the plasma samples under testing or of the various concentrations of the PAI-1-mVn complex standards (final concentration of PAI-1 in the PAI-1/mVn complex range from 0 to 100ηg/ml) are added to each